JOURNAL OF CLINICAL MICROBIOLOGY, July 1995, p. 1739–1741 Vol. 33, No. 7
0095-1137/95/$04.00+0

Identification of Microsporidia in Stool Specimens by Using PCR and Restriction Endonucleases

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Received 23 January 1995/Returned for modification 28 March 1995/Accepted 17 April 1995

We report the development of a PCR-based assay for the detection of microsporidia in clinical specimens. A single primer pair complementary to conserved sequences of the small-subunit rRNA enabled amplification of DNA from the four major microsporidian pathogens of humans: *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, *Enterocytozoon bieneusi*, and *Septata intestinalis*. The extraction method allowed PCR amplification of *E. bieneusi* and *S. intestinalis* DNA from sodium hypochlorite-treated stool specimens. Differentiation of the microsporidian gastrointestinal pathogens *E. bieneusi* and *S. intestinalis* could be accomplished by restriction endonuclease digestion of PCR products using *Pst*I and *Hae*III.

Microsporidia are obligate intracellular parasites infecting both invertebrates and vertebrates. Five genera of microsporidia have been reported as etiologic agents of disease in humans. *Encephalitozoon cuniculi*, *Nosema* spp., and *Pleistophora* spp. cause a wide variety of infections, primarily in immunocompetent hosts. Infections with *Encephalitozoon hellem*, *Enterocytozoon bieneusi*, and *Septata intestinalis* have been identified in patients with AIDS; the last two organisms appear to be at least partially responsible for the chronic diarrhea and wasting syndrome observed in these patients (13). Unclassified microsporidian organisms are referred to by the collective term "microsporidium." A recent report indicates that infection with *E. bieneusi* is not limited to human immunodeficiency virus-positive patients but can also cause a self-limited diarrheal syndrome in immunocompetent individuals (8). No controlled trials of therapeutic modalities for microsporidian infection have thus far been conducted; however, case reports indicate that albendazole may be curative for infections caused by *S. intestinalis* but not by *E. bieneusi* (1, 2, 4, 6).

Diagnosis of gastrointestinal microsporidiosis can be made by detecting spores in stool specimens with Weber's or Ryan's modified trichrome stain or optical brightening agents such as Uvitex 2B (3, 7, 10, 12). Confirmation of infection is made by demonstration of organisms in intestinal biopsy specimens by light or transmission electron microscopy (12, 13). Identification of microsporidia to the species level is dependent currently upon observation of characteristic intracellular morphology when viewed in the electron microscope. PCR has been used to detect and identify microsporidia; however, these studies have been limited to amplification of DNA from biopsy specimens, and all previous investigators have used species-specific primers in their PCR assays (11, 15). This paper describes a PCR assay utilizing a primer pair that will amplify all four major microsporidian pathogens, *E. cuniculi*, *E. hellem*, *E. bieneusi*, and *S. intestinalis*, and further demonstrates its ability to amplify DNA extracted from stool specimens. A simple digestion of PCR products with restriction endonucleases can be performed to provide a species identification of the microsporidian gastrointestinal pathogens *E. bieneusi* and *S. intestinalis*.

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MATERIALS AND METHODS

Parasite cultures. *E. cuniculi* JS was originally obtained from Elizabeth Didier, Tulane University. *E. hellem* CDC:0291:V213, *S. intestinalis* CDC:V297, and the E6 (monkey kidney) cell line were originally obtained from Govinda S. Visvesvara, Centers for Disease Control and Prevention. All microsporidian parasites were cultivated in E6 cells with medium formulations originally provided by Govinda S. Visvesvara. E6 growth medium consisted of 500 ml of RPMI 1640 (BioWhittaker, Walkersville, Md.), 35 ml of heat-inactivated fetal bovine serum (BioWhittaker), 5 μg of epidermal growth factor stock (Sigma, St. Louis, Mo.), 1 ml of insulin-transferrin-sodium selenite medium supplement (Sigma), 0.036 μg of water-soluble hydrocortisone (Sigma), and 5 ml of 100× penicillin-streptomycin-amphotericin B (Fungizone) mix (BioWhittaker). Medium for infected cells consisted of 500 ml of RPMI 1640, 15 ml of fetal bovine serum, 1 ml of insulin-transferrin-sodium selenite medium supplement, and 5 ml of 100× penicillin-streptomycin-amphotericin B mix. Parasite spores were harvested weekly and were washed and resuspended in 50 mM (pH 7.2) phosphate-buffered saline (PBS) (16).

Stool specimens. Stool specimens were obtained from human immunodeficiency virus-positive outpatients at the Warren Grant Magnuson Clinical Center, National Institutes of Health, Bethesda, Md. Microsporidia were detected in stool specimens with a modification of the chromotrope 2R stain (modified trichrome stain; Remel, Lenexa, Kan.) as described by Weber et al. (12), except that methanol-fixed stool smears were stained with the modified trichrome stain for 30 min at 37°C.

DNA extraction. Diluted stool specimens (1:1 in 0.5% sodium hypochlorite) were centrifuged at 15,000 × *g* for 5 min, and the pellet was washed three times with PBS. The washed pellet was resuspended in 200 μl of lysis buffer (10 mM Tris · HCl, 100 mM of NaCl, 20 mM dithiothreitol, 2 mg of proteinase K per ml, 250 U of lyticase [Sigma] [pH 8.0] per ml). After 15 min of incubation, mechanical disruption was performed with 425- to 600-μm-diameter glass beads (Sigma) and a mini-bead beater (Biospec Products) for 2 min. Extracts were then incubated at 37°C for 18 h, 150 μl of 2% sodium dodecyl sulfate and 200 μl of proteinase K (2 mg/ml) were added, and incubation was continued at 50°C for 72 h. DNA was phenol-chloroform extracted, purified with the Geneclean II kit (Bio 101, Inc., La Jolla, Calif.), and resuspended in 30 μl of TE buffer (10 mM Tris · HCl, 1 mM EDTA [pH 7.4]). Cultured organisms were washed twice in PBS, and the DNA was extracted by using bead beating, phenol-chloroform, and ethanol precipitation.

PCR amplification. Primers for PCR were chosen to amplify a conserved region of the small-subunit rRNA of *E. cuniculi*, *E. hellem*, *S. intestinalis*, and *E. bieneusi*. The forward primer (5′-CACCAGGTTGATTCTGCCTGAC-3′), complementary to positions 1 to 22, was designed by using a published sequence of *E. bieneusi* (5) and GenBank sequences of *E. cuniculi* (accession number L17072), *E. hellem* (accession number L19070), and *S. intestinalis* (accession number U09929). The reverse primer was designed to be complementary to positions 230 to 250 of a published sequence of *E. bieneusi* (5) and GenBank sequences of positions 248 to 268 of *E. cuniculi* (accession number L17072), 259 to 279 of *E. hellem* (accession number L19070), and 250 to 270 of *S. intestinalis* (accession number U09929). Two primers were selected for evaluation: PMP2 (5′-CCTCTCCGGAACCAAACCCTG-3′) and PMP2b (5′-CCTCTCCGGA ATCAAACCCCG-3′). PCR was performed using 2.5 μl of DNA in a final volume of 50 μl with the GenAmp kit (Perkin-Elmer Cetus, Norwalk, Conn.) according to the manufacturer's instructions and a Perkin-Elmer 9600 thermocycler. A hot-start procedure was used employing Ampliwax PCR GEM 100

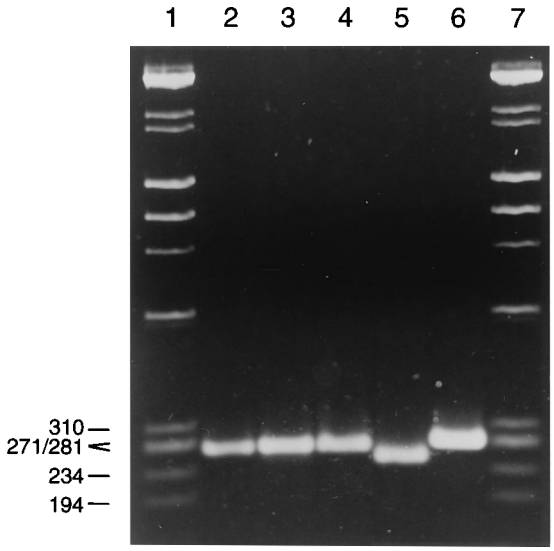

FIG. 1. Agarose gel electrophoresis of PCR amplification products from microsporidia. Lanes: 1 and 7, molecular weight markers (*Hae*III digest of ϕX174 [Bethesda Research Laboratories]); 2, *E. cuniculi* from culture; 3, *S. intestinalis* from culture; 4, *S. intestinalis* from stool specimen; 5, *E. bieneusi* from stool specimen; 6, *E. hellem* from culture. Sizes (in nucleotides) of relevant markers are indicated on the left.

beads (Perkin-Elmer Cetus) with an initial denaturation at 94°C for 10 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final hold at 72°C for 10 min for primer extension. PCR products were detected by agarose gel electrophoresis and ethidium bromide staining.

Restriction endonuclease digestion of PCR products. *Pst*I and *Hae*III restriction endonucleases (New England Biolabs, Inc., Beverly, Mass.) were used to digest PCR products obtained from amplification of culture and stool DNA extracts. Five microliters of PCR product was digested with 20 U of *Pst*I or 10 U of *Hae*III in a final volume of 15 μl, and products were detected by agarose gel electrophoresis and ethidium bromide staining.

DNA sequencing. To confirm the identity of organisms in stool specimens, microsporidian small-subunit RNA gene fragments were amplified with primers PMP1E (5′-CTCGAATTCCACCAGGTTGATTCTGCCTGAC-3′) and PMP2H (5′-CTCAAGCTTCCTCTCCGGAACCAAACCCTG-3′), and the products were digested with *Eco*RI and *Hin*dIII prior to being subcloned into the complementary sites within the multiple cloning site of the plasmid vector pTZ19-R (Pharmacia, Alameda, Calif.). For each specimen, the nucleotide sequence of inserts from three independent plasmid subclones was determined by a commercial company (Lofstrand Laboratories, Gaithersburg, Md.), using a standard dideoxy chain termination procedure, and then compared with published sequences.

RESULTS

DNA was easily extracted from cultured organisms, but microsporidian spores in stool specimens required harsh conditions employing both mechanical and chemical disruption and a laborious 4-day procedure. A dilution series using stool specimens spiked with *E. cuniculi* DNA demonstrated that fresh stool strongly inhibited the PCR assay but treatment of stool with 0.5% sodium hypochlorite or 10% formalin removed the PCR inhibition (data not shown). Although formalin was somewhat more efficient at removing the PCR inhibition than was sodium hypochlorite, the extraction method used in this study did not result in efficient extraction of DNA from formalin-fixed stool specimens, and thus, sodium hypochlorite-treated specimens were used.

Use of reverse primer PMP2 resulted in efficient amplification of all organisms tested (Fig. 1); primer PMP2b failed to amplify *E. bieneusi* and thus was not further investigated. PCR of DNA from each microsporidian parasite resulted in amplicons of slightly different sizes (Fig. 1). Amplicon sizes were 250 bp for *E. bieneusi*, 268 bp for *E. cuniculi*, 270 bp for *S. intestinalis*, and 279 bp for *E. hellem*. Digestion of amplicons using *Pst*I and *Hae*III produced distinctive fragments detectable in ethidium bromide-stained agarose gels (Fig. 2; Table 1). An important observation was that *E. bieneusi* does not have a *Pst*I restriction site in the amplified region but *Pst*I will cut *S. intestinalis* into two distinct fragments.

The sequences of amplified DNA fragments amplified from stool samples matched the sequences of GenBank database accession numbers L16868 (*E. bieneusi*) and U09929 (*S. intestinalis*), indicating that we successfully amplified both *E. bieneusi* and *S. intestinalis* from the stools of infected patients. Cultured *S. intestinalis* was added to a stool specimen positive

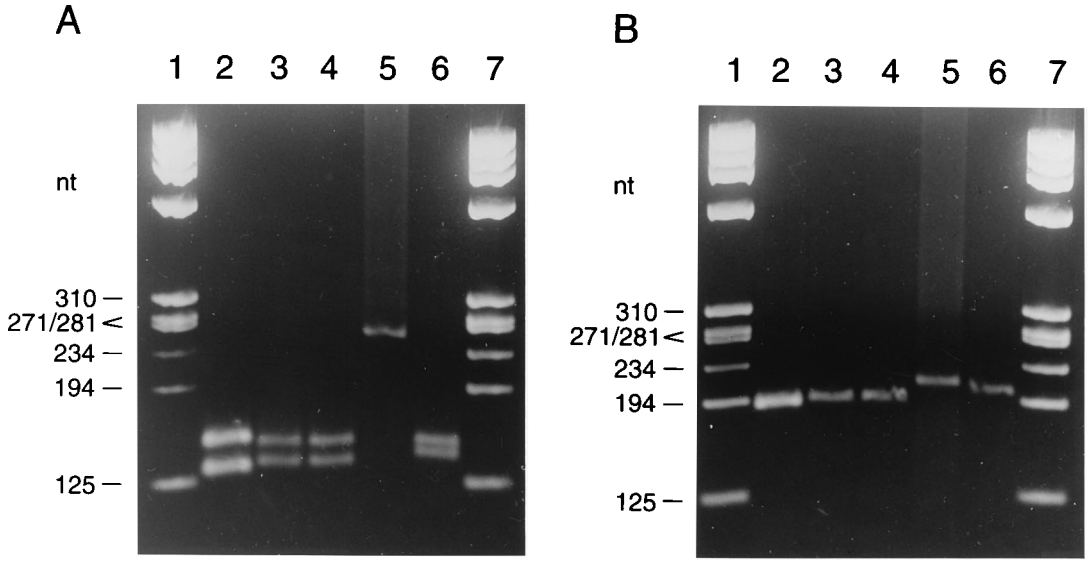

FIG. 2. Agarose gel electrophoresis of results from restriction endonuclease digestion of PCR products. (A) *Pst*I; (B) *Hae*III. Lanes: 1 and 7, molecular weight markers (*Hae*III digest of ϕX174 [Bethesda Research Laboratories]); 2, *E. cuniculi* from culture; 3, *S. intestinalis* from culture; 4, *S. intestinalis* from stool specimen; 5, *E. bieneusi* from stool specimen; 6, *E. hellem* from culture. Sizes (in nucleotides [nt]) of relevant markers are indicated on the left.

TABLE 1. Sizes of PCR products and restriction enzyme digests

Organism	Size(s) (bp) of:		
	PCR product	*Pst*I fragment(s)	*Hae*III fragment
E. bieneusi	250	250	208
E. cuniculi	268	122, 146	189
S. intestinalis	270	124, 146	193
E. hellem	279	133, 146	200

for *E. bieneusi* to simulate a dual infection. PCR followed by *Pst*I digestion enabled the presence of both organisms to be clearly demonstrated (data not shown).

DISCUSSION

Examination of sequences deposited in GenBank for the gene encoding the small-subunit rRNA of *E. cuniculi*, *E. hellem*, *E. bieneusi*, and *S. intestinalis* reveals considerable disagreement about the degree of sequence conservation in the 5′ region (bp 1 to 300) of this gene. Since DNA from all four parasites was efficiently amplified in our assay using the PMP2 reverse primer sequence, we conclude that the region covered by this primer is conserved in these organisms and, therefore, a single PCR using primer pair PMP1 and PMP2 can enable diagnosis of infections caused by all four microsporidia investigated. Use of the PMP1-PMP2 primer pair and *Pst*I and *Hae*III restriction endonucleases enabled ready differentiation of *E. bieneusi* and *E. hellem* from *S. intestinalis* and *E. cuniculi* but did not allow the differentiation of *S. intestinalis* from *E. cuniculi* (Fig. 2). Since only *E. bieneusi* and *S. intestinalis* are presently recognized as enteric pathogens (13), our PCR assay may obviate the need for laborious electron microscopic examination of biopsy material or stool specimens to determine the species of microsporidia responsible for gastrointestinal infection. Although light or electron microscopic examination of biopsy specimens may still be required to confirm microsporidian disease, PCR allows the identification of species of microsporidia in patient specimens.

A simulated dual infection with both *E. bieneusi* and *S. intestinalis* was detected by our PCR assay. The existence of such dual infections was demonstrated by Van Gool et al. when they attempted to culture *E. bieneusi* from biopsy-proven positive patients (9). *S. intestinalis* was recovered from seven stool samples obtained from four patients but could not be detected in biopsy specimens, even after extensive examination. *E. bieneusi* spores measure 1 to 1.5 μm and *S. intestinalis* spores measure 2 to 2.5 μm, but the difference in size between the two organisms is not sufficient to allow detection of a dual infection or identification of organism species from light microscopic examination of specimens, suggesting that PCR may therefore become the method of choice for identification of microsporidia in clinical specimens.

Microsporidian species appear to differ in their susceptibilities to antimicrobial agents. Albendazole has been reported to be parasiticidal against *S. intestinalis* but appears to be only parasitistatic against *E. bieneusi* (1, 2, 4, 14). When more information concerning the in vivo responses of microsporidia to antimicrobial agents is available, rapid identification of intestinal microsporidia to the species level by PCR may help physicians decide on treatment options. PCR may also find utility as a means of monitoring the effectiveness of antimicrosporidian therapy.

This is the first report of a procedure utilizing PCR and restriction endonuclease digestion for detection and identification of species of microsporidia from stool specimens. The current procedure uses sodium hypochlorite to block PCR inhibitors present in stool specimens. Since 10% formalin efficiently eliminates PCR inhibitors from stool and is a widely used preservative for parasitological examination of stool specimens, future work will focus on developing an efficient method to extract microsporidian DNA from formalin-fixed stool specimens.

ACKNOWLEDGMENT

We thank Graham Clark from the National Institute for Allergy and Infectious Diseases for providing E6 cells, *E. hellem*, *S. intestinalis*, and the medium formulations used to grow them.

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